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(54) Title: SENSITIVE DETECTION OF CELL SURFACE MARKERS ON VIABLE CELLS

(57) Abstract

Magnetofluorescent liposomes are size selected by membrane extrusion and magnetic separation. The selection process provides a highly fluorescent population of liposomes, which are used as a fluorescent labeling agent. Intensity of fluorescent staining can be increased 100-1000-fold without increased background fluorescence, when compared to conventional fluorochrome-conjugated antibodies. The highly sensitive staining permits detection of cell surface markers expressed at low levels on the cell surface. Of particular interest is the detection of normally secreted certain cytokines, e.g. interferon γ, and IL-10 on the surface of lymphocytes. Cells that are positive for surface expression of these cytokines may be quantitated or separated for further use.

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SENSITIVE DETECTION OF CELL SURFACE MARKERS ON VIABLE CELLS

INTRODUCTION

Technical Field

The field of this invention is detection and isolation of cells according to surface phenotype.

Background

Immunofluorescence and immunomagnetism have found widespread recognition for specific analysis and separation of cells. Detection by these techniques is dependent on the number of antigens per cell, the molar ratio of fluorochrome to antibody, the cellular autofluorescence and the sensitivity of the instrument. The sensitivity of staining with antibody fluorochrome conjugates is limited in principle since only a relatively small number of dye molecules can be conjugated to a single antibody for detection, but a relatively large number of fluorescent molecules is required to obtain a detectable signal.

A generally accepted value for the detection limit of fluorescent is 3000-5000 molecules per cell, if cellular autofluorescence is low. This corresponds to at least 1000 antibodies bound. With increasing cellular autofluorescence, classical immunofluorescence becomes even less sensitive. Thus, antigens expressed in low copy number per cell are not accessible to cytometric analysis, although they may have functional importance. Additionally, these molecules may only be expressed by a very small subpopulation of cells in the peripheral blood or bone marrow (<1%). Thus, analysis is complicated or even impossible without preanalytical magnetic enrichment. It is therefore of interest to develop reagents and methods that can be used to detect markers present in low numbers on the surface of cells.

The differentiation of cytokine expression in T lymphocytes is a focus of immunological research at present, with the aim to identify and analyze cellular

and molecular parameters decisive for selection of particular immune reactions. A powerful method to identify the cytokines expressed by individual T cells is intracellular immunofluorescence, which stains nascent cytokine proteins en route to secretion. For this analysis, however, cells have to be fixed and permeabilised, which kills the cells and disables further functional analysis.

T cells exert their central role in the control of immune responses via membrane bound ligands or secreted cytokines, e.g. IL-2, IL-4, IL-5, IL-10 and IFN-γ. Among these, IFN-γ, and IL-10 are antagonistic molecules, providing distinct help for inflammatory and humoral immune responses. They are expressed by Th1 and Th2 cells, respectively. Several attempts have been made to identify surface markers distinctive for live Th1 and Th2 cells, but the results have been ambiguous. Methods that can be used to separate viable lymphocytes are of interest for the study of immune system regulation.

Relevant Literature

The conjugation of antibodies to fluorescein-filled liposomes is described in Loughrey et al. (1990) J. Immunol. Methods **132**:25-35; Schwendener et al. (1990) Biochim Biophys Acta **1026**:69-79; Truneh et al. (1987) J. Immunol. Methods **100**:59-70; and Leserman et al. (1980) Nature 288:602-604. A five to ten fold improvement of signal to noise ratio over fluorescein conjugated antibodies is shown in Gray et al. (1990) J. Immunol. Methods **121**:1-7; Gray et al. (1992) Ann. Biol. Clin. **50**:169-174; and Trunch and Machy (1987) Cytometry **8**:562-567. Extrusion of liposomes through polycarbonate membranes with defined pore size is disclosed in Hope et al. (1985) Biochim. Biophys. Acta **812**:55-65; and Olson et al. (1981) Biochim. Biophys. Acta **557**:9-23. Dextran-magnetite-incorporated thermosensitive liposomes are described in Viroonchatapan (1995) Pharm. Res. **12**:1176-1183.

An analysis of intracellular cytokines is provided by Assenmacher et al. (1994) Eur. J. Immunol. **24**:1097; and Prussin and Metcalfe (1995) J. Immunol. Methods **188**:117-128. The specific expression of surface interferon-gamma on interferon-gamma producing T cells is described in Assenmacher et al. (1996) Eur J Immunol **26**:263-7. The detection of surface interferon-γ, was reported by

Caruso et al. (1988) J. Immunol. Methods 113:37-43. The analysis and sorting of live cells according to secreted molecules relocated to a cell-surface affinity matrix is described in Manz et al. (1995) P.N.A.S. 92:1921-5. An artificial affinity matrix, specific for the secreted product of interest, is created on the cell surface, and the cells are allowed to secrete for a defined time period. The secreted molecules bind to the affinity matrix on the secreting cell and are subsequently labeled with specific staining reagents.

Fluorescence-activated cytometry cell sorting based on immunological recognition is reviewed in Assenmacher et at. (1995) Clin Biochem 28:39-40. The detection and isolation of rare cells is reviewed in Radbruch and Recktenwald (1995) Curr. Opin. Immunol. 7:270-3. High gradient magnetic cell sorting is described in Miltenyi et al. (1990) Cytometry 11:231-238. Molday, U.S. 4,452,773 describes the preparation of magnetic iron-dextran microspheres and provides a summary describing the various means of preparation of particles suitable for attachment to biological materials.

SUMMARY OF THE INVENTION

Methods and compositions are provided for sensitive detection of cell surface markers. Unilamellar liposomes are loaded with magnetic microparticles and a fluorochrome, then size selected by membrane extrusion and magnetic separation. The magnetic separation enriches for larger liposomes, which have enclosed a magnetic microparticle, thereby depleting small, undesirable liposomes. The liposomes are then used as a fluorescent labeling agent, and may be conjugated to specific binding agents, e.g. nucleic acids, antibodies, etc. Intensity of fluorescent staining can be increased 100-1000-fold without increased background fluorescence, when compared to conventional fluorochrome-conjugated antibodies.

The highly sensitive staining permits detection of cell surface markers expressed at low levels on the cell surface. Of particular interest is the detection of certain normally secreted cytokines, e.g. interferon γ and IL-10, on the surface of lymphocytes. Cells that are positive for surface expression of these cytokines may be quantitated or separated for further use.

Brief Description of the Drawings

Figures 1A and 1B are graphs showing the fluorescence intensity distribution of liposomes after several extrusion cycles through polycarbonic membranes of 0.4 μm pore size. Figure 1A: unseparated liposome fraction. Figure 1B: magnetic liposome fraction after elution from a MACS column.

Figures 2A to 2E are plots showing the staining of IFN-γ on the surface of live murine T cells.

Figures 3A and 3B show surface IFN-γ, staining on human T cells.

Figures 4A to 4D show the correlation of surface and intracellular IFN-γ expression by the same cells.

Figure 5 is a bar graph showing cytokine secretion by murine T cells sorted for surface IFN-γ expression.

Figures 6A to 6D show the expression of surface and intracellular IFN-γ, by murine IFN-γ receptor negative T cells.

Figures 7A to 7C show the expression of surface IL-10 on the surface of activated murine T cells.

Figures 8A to 8D show the intracellular IL-10 expression of murine T cells after sorting for surface IL-10.

Figure 9 is a bar graph showing cytokine secretion by murine T cells sorted for surface IL-10 expression.

Description of the Specific Embodiments

Methods and compositions are provided for sensitive labeling of specific binding agents. Unilamellar liposomes are formed in the presence of magnetic microparticles and a fluorochrome. The liposomes are selected by extrusion through a membrane of defined pore size to provide an upper limit on size. They are then magnetically selected to enrich for liposomes that have incorporated a magnetic microparticle. The magnetic separation enriches for larger liposomes, thereby depleting small liposomes that interfere with subsequent staining. The magnetofluorescentliposomes are conjugated to specific binding agents, e.g. nucleic acids, antibodies, etc. and used as a labeling agent. Intensity of fluorescent staining is significantly improved over the staining observed with

unselected liposomes, or with fluorochrome conjugated antibodies. The presence of the magnetic microparticle also allows for magnetic selection of the labeled binding agents.

The high fluorescence of the labeled binding agents permits very sensitive detection of markers, e.g. antigens, expressed at low levels on a cell surface. An exemplary use is the detection of certain normally secreted cytokines, e.g. interferon γ, (IFN-γ,) and interleukin 10 (IL-10), on the surface of lymphocytes. Using the subject reagents, it has been shown that trace levels of these cytokines are present on the surface of T lymphocytes. Expression of IFN-γ, is characteristic of Thl type T cells, and IL-10 expression is characteristic of Th2 type T cells. Cells that are positive for surface expression of these cytokines may be quantitated or separated for further use, thus permitting isolation of viable Thl and Th2 cell subsets.

The subject large unilamellar liposomes encapsulate fluorochromes and a magnetic microparticle. The liposomes are formed by methods known in the art, for example, as described by Hope et at. (1985) Biochim Biophys Acta **812**:55-65; Karo et al. (1991) J. Biol. Chem. **266**:3361; etc. The lipids may be any useful combination of known liposome forming lipids, including cationic lipids, such as phosphatidylcholine, dipalmitoylphosphatidyl ethanolamine, cholesterol, phosphatidyl serine, phosphatidyl glycerol, soy phosphatidylcholine, and the like. Optionally, the liposomes are functionalized for a conjugation step by the inclusion of lipophilic maleimide compounds, e.g. N-(3-maleimidopropionyl)-N2-palmitoyl-L-lysine methyl ester (MP-PL), N-(3-maleimidopropionyl) phosphatidylethanolamide (MP-PE), N6-(6-maleimidocaproyl)-N2-palmitoyl-L-lysine methyl ester (EMC-PL), N-(6-maleimidocaproyl) phosphatidylethanolamine (EMC-PE), etc. Alternatively, lipids may by modified with a thio group, e.g. N-[3-(2-pyridildithio)-propionyl] dipalmitoylphosphatidyl ethanolamine to provide a reactive moiety for conjugation.

To load the liposomes, the lipids are hydrated in an appropriate aqueous medium containing the fluorochrome at a concentration of from about 1 to 100 mM; and superparamagnetic microparticles, at a concentration of from about 1 to 10% vol/vol. The superparamagnetic microparticles are described in Molday,

supra. and Miltenyi et al. (1990) supra. The microparticles will usually have a diameter of at least about 50 nm, and less than about 200 nm, usually less than about 100 nm.

Suitable fluorochromes include fluorescein isothiocyanate (FITC); rhodamine and derivatives thereof; Texas Red; phycoerythrin; allophycocyanin; 6-carboxyfluorescein (6-FAM); carboxyfluorescein (CF); 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE); 6-carboxy-X-rhodamine (ROX); 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX); 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA); N,N'-bis(1-hexylhfetyl)-3,4:9,10-perylenebis(dicarboximid) (BHPD); etc.

Liposomes are formed by subjecting the suspension to freeze-thaw cycles, sonication, vortexing, etc. The liposomes are then extruded through one or more filters, e.g. polycarbonate, glass fiber, cellulose, nitrocellulose, etc. having pores of a defined size. Generally filters having a larger pore size, of less than about 1 pm, usually about 800 nm, will be used first. The extrusion may be performed from one to about ten or more times. The final extrusion(s) use a filter having a pore size of around about 400 nm. The liposome composition after filter extrusion will have an upper size limit defined by the pore size, but will be heterogeneous as to small particle size, i.e. will range in size from about 50 to 400 nm in diameter. The liposomes may be separated from free magnetic particles according to density, e.g. sucrose gradients, ficoll density gradients, etc.

Liposomes containing magnetic particles are isolated from the smaller, non-magnetic liposomes by high gradient magnetic field filtration. A suspension of magnetically labeled cells is applied to a column or chamber as described in U.S. 5,385,707, herein incorporated by reference. The matrix may consist of closely packed ferromagnetic spheres, steel wool, wires, magnetically responsive fine particles, etc. The matrix is composed of a ferromagnetic material, e.g. iron, steel, etc. and may be coated with an impermeable coating to prevent the contact of liposomes with metal. The matrix should have adequate surface area to create sufficient magnetic field gradients in the separation chamber to permit efficient retention of magnetically labeled liposomes. The volume necessary for a given

separation may be empirically determined, and will vary with the cell size, antigen density on the cell surface, cell number, antibody affinity, etc.

The liposomes are bound to the matrix in the presence of a magnetic field, usually at least about 100 mT, more usually at about 500 mT, usually not more than about 2T, more usually not more than about 1T. The source of the magnetic field may be a permanent or electromagnet. The unbound liposomes contained in the eluate are discarded.

The bound liposomes are released by removing the magnetic field, and eluting in a suitable buffer. The liposomes may be collected in any appropriate medium which maintains the integrity of the liposomes, e.g PBS, normal saline, aqueous buffers, etc. After the magnetic separation, the liposomes are substantially homogenous in size, of at least about 100 nm in diameter, usually at least about 200 nm, and less than about 500 nm in diameter, usually less than about 400 nm.

The magnetofluorescent liposomes are conjugated to a specific binding agent through any convenient method. Where a lipophilic maleimide compound is included in the liposome, coupling agents such as SPDP (N-succinimidyl-3-(2-pyridyldithio)propionate); SATA (N-succinimidyI-S-acetylthioacetate); 4-(N-maleimidomethyl)-cyclohexane-1-carboxylic acid N-hydroxysuccinimide ester (SMCC); etc. may be used to couple the maleimide moiety to a reactive sulfhydryl group on the binding agent. Alternatively, the binding agent, e.g. antibody, may be treated with iminothiolane and reacted with a functionalized lipid such as N-[3-(2-pyridildithio)-propionyl] dipalmitoyl phosphatidyl ethanolamine.

The term "specific binding agent" as used herein refers to a member of a specific binding pair, i.e. two molecules, usually two different molecules, where one of the molecules through chemical or physical means specifically binds to the other molecule. The complementary members of a specific binding pair are sometimes referred to as a ligand and receptor. In addition to antigen and antibody specific binding pairs, peptide-MHC antigen and T cell receptor pairs; alternative specific binding pairs of interest include biotin and avidin or streptavidin; carbohydrates and lectins; complementary nucleotide sequences (including nucleic acid sequences used as probes and capture agents in DNA

hybridization assays); peptide ligands and receptor; effector and receptor molecules; hormones and hormone binding protein; enzyme cofactors and enzymes; enzyme inhibitors and enzymes; secretion markers, as described in International application PCT/US93/10126; autologous monoclonal antibodies, and the like. The specific binding pairs may include analogs, derivatives and fragments of the original specific binding member. For example, an antibody directed to a protein antigen may also recognize peptide fragments, chemically synthesized peptidomimetics, labeled, derivated, etc. so long as an epitope is present.

Immunological specific binding pairs include antigens, e.g. proteins, peptides, carbohydrates, etc., and haptens, specific antibodies or T cell antigen receptors. Recombinant DNA methods or peptide synthesis may be used to produce chimeric, truncated, or single chain analogs of either member of the binding pair, where chimeric proteins may provide a mixture(s) or fragment(s) thereof, or a mixture of an antibody and other specific binding members. Antibodies and T cell receptors may be monoclonal or polyclonal, and may be produced by transgenic animals, immunized animals, immortalized human or animal B-cells, cells transfected with DNA vectors encoding the antibody or T cell receptor, etc. The details of the preparation of antibodies and their suitability for use as specific binding members are well-known to those skilled in the art.

For brevity, the detection and separation with the subject binding agents will mainly be described in terms of its ability to specifically select and separate a defined population of cells (target cells) from a mixed cell population, such as peripheral blood, bone marrow, blood from the umbilical cord or placenta, fetal blood or a leukapheresis product. It will also be appreciated that some tissues may be disrupted into a single cell suspension to allow isolation of a particular cell subset, such as the separation of tumor infiltrating lymphocytes from a tumor mass. For example, different cell types may be labeled with a specific antibody to allow cell purging and/or cell enrichment. The target cell population is generally identified by a specific binding member as described above, that selectively binds to a cell surface antigen present on the target cells. It should be understood, however, that the subject compositions and methods is not limited to such uses.

For simplicity, the specific binding member will be exemplified herein by an antibody. The antibody may be directly or indirectly bound to an magnetofluorescent liposome (MFL). If the antibody is directly bound to the MFL, then the target cell population is labeled when the antibody binds to the cell surface antigen. If the antibody is indirectly bound to the MFL, then the target cell population is susceptible to labeling when the antibody is bound to the target cells. The antibody-bound cell population is labeled by further contacting the cells with a specific binding member for the antibody, where that specific binding member is itself bound to a magnetic particle. For example, a specific binding member such as avidin can be conjugated to an MLV wherein the avidin binds to a biotinylated antibody which in turn specifically binds to the target cells. The MFL may be conjugated to an antibody specific for a hapten, e.g. digoxin, digoxigenin, FITC, dinitrophenyl, nitrophenyl, etc., where a second antibody specific for a cell surface determinant is then conjugated to the hapten. Methods for conjugation of haptens to antibody are known in the art.

A sample of cells is prepared for labeling. The sample may be subjected to prior treatment, such as dilution in buffered medium, concentration, filtration, or other gross treatment that will not involve any specific separation. Biological samples containing significant numbers of red blood cells may be treated by conventional methods to prevent clotting, such as the addition of EDTA, heparin or acid-citrate-dextrose solution. A preparation of nucleated cells may be made from the sample, using conventional procedures, e.g. Ficoll-Paque density gradients, elutriation, resuspension in a solution that lyses erythrocytes, e.g. ammonium chloride-potassium; ammonium oxalate, etc.

The subject labeled antibodies are added to the cell sample. Especially useful are antibodies specific for cell surface antigens. Of particular benefit are antibodies specific for antigens that are expressed at very low levels on the cell surface, e.g. IFN-γ, IL-10, CD25, hormone or cytokine receptors, Thy-1 on certain cell populations, T cell receptor on certain cell populations, surrogate immunoglobulin light chain, etc. Whole antibodies may be used, or fragments, e.g., Fab, F(ab')2, light or heavy chain fragments, etc. Such antibodies may be polyclonal or monoclonal and are generally commercially available or

alternatively, readily produced by techniques known to those skilled in the art. Useful antibodies will have a low level of non-specific staining, and will usually have an affinity of at least about 100 μM for the antigen.

The antibodies are added to a suspension of cells, and incubated for a period of time sufficient to bind the available cell surface antigens. The incubation will usually be at least about 5 minutes and usually less than about 30 minutes. It is desirable to have a sufficient concentration of antibodies in the reaction mixture, so that the efficiency of the magnetic separation is not limited by lack of antibody. The appropriate concentration is determined by titration. The medium in which the cells are separated will be any medium which maintains the viability of the cells. A preferred medium is phosphate buffered saline containing from 0.1 to 0.5% BSA. Various media are commercially available and may be used according to the nature of the cells, including Dulbecco's Modified Eagle Medium (dMEM), Hank's Basic Salt Solution (HBSS), Dulbecco's phosphate buffered saline (dPBS), RPMI, Iscove's medium, PBS with 5 mM EDTA, etc., frequently supplemented with fetal calf serum, BSA, HSA, etc.

Where an indirectly labeled antibody is used, the cell suspension may be washed and resuspended in medium as described above prior to incubation with the second stage antibodies. Alternatively, the second stage antibody may be added directly into the reaction mix. When directly coupled depletion antibodies are used, the cell suspension may be used directly in the next step, or washed and resuspended in medium.

Analysis of antibody binding will preferably use a sensitive detection system, e.g. laser detection by flow cytometry, sensitive fluorescence microscopy, particularly in combination with automated image analysis (see, for example, U.S. Patent Nos. 4,741,043; 5,109,429 and 5,202,931). Separation of labeled cells may utilize the fluorescent label, through fluorescence activated cell sorting, or may take advantage of the magnetic label to separate by high gradient magnetic separations (Miltenyi et al. (1990) supra.) After the separation is complete, the cells are used as appropriate. The method of harvesting will depend on the type of analysis to be performed.

In order to address the needs of research and clinical laboratories, a kit may be provided having the reagents and apparatus necessary to perform the subject invention. Such a kit will contain magnetofluorescent liposomes. The liposomes may be provided already conjugated to affinity reagents, e.g. antibodies specific for cell markers, anti-hapten antibodies, anti-Ig antibodies, etc. When the microparticles are conjugated to second stage antibodies, suitable first stage antibodies may also be provided.

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1

Preparation of Magnetofluorescent Liposomes

Materials and Methods

Antibodies and preparation of conjugates. Pure protein and fluorescein-conjugated sheep anti-digoxigenin (Fab)2 fragments were purchased from (Boehringer Mannheim, Mannheim, Germany); antibodies against CD3 (clone SK7), 6D19 (clone 4G7) and 6D25 (clone 2A3) were obtained from Becton Dickinson & Co., (San Jose, CA), digoxigenin (Dig)-conjugation was done with the Dig antibody labelling kit from Boehringer Mannheim; phycoerythrin (PE) conjugation was done following conventional protocols (Mueller (1992) in Flow Cytometry and Cell Sorting, Springer Verlag, Berlin, pp. 27-33).

Reagents. DPPC, DPPG and DPPE were gifts from Lipoid KG (Mannheim, Germany); cholesterol was purchased from Sigma (Deisenhofen, Germany); SPDP was obtained from Pierce (Oud-Beijerland, The Netherlands), triethylamine and 2-iminothiolan were purchased from Fluka (Buchs, Switzerland) and used without further purification; magnetic beads were prepared as described by Molday et al. (1982) Immunol. Meth. 52:353-368, and provided by Miltenyi Biotec.

Synthesis of N-[3-(2-pyridildithio)-propionyl] dipalmitoylphoslahatidyl ethanolamine (PDP-DPPE). PDP-DPPE was synthesized according to Leserman

et al. (1980) Nature 288:602-604. Briefly, to 50 mmol DPPE dissolved in 3 ml of chloroform/methanol (3:1 v/v) 100 mmol of triethylamine and 100 mmol of SPDP dissolved in I ml of chloroform were added. The reaction mixture was stirred at room temperature for several hours, until analysis by thin layer chromatography on silica plates (TLC, running solvent: chloroform/methanol/water, 65:25:4) indicated complete conversion of DPPE to a faster running product. The mixture was washed subsequently with 1 mM HCl and water until the aqueous phase reacted neutral. The purified reaction product, which runs as a single spot in TLC, was stored in chloroform at -20°C.

Preparation of liposomes. Large unilamellar vesicles (LUVs) were prepared as described by Hope et at. (1985) Biochim Biophys. Acta 812:55-65. Briefly, 300 μmol of mixture of lipids (molar ratio DPPC:Chol:DPPG:PDP-DPPE = 45:40:10:5) dissolved in chloroform and glass beads were deposited in a glass vessel. The solvent was allowed to evaporate and the remaining lipid film was dried under vacuum for 1 h at 65°C. The lipids were then hydrated at 65°C with about 4 ml of PBS, pH 7.5, containing 10 mM CF and magnetic beads. The suspension was submitted to several freeze-thaw cycles and extruded five to ten times through filters of 800-and, subsequently, 400-nm pore size (Millipore, Eschborn, Germany). Liposomes were separated from free magnetic particles by centrifugation over a Ficoll (Pharmacia, Freiburg, Germany) density gradient. Liposomes containing magnetic particles were then isolated by high gradient magnetic field filtration, with the magnetic cell separation system MACS (Miltenyi Biotec, Bergisch Gladbach, Germany). Size distribution was estimated by measuring the relative fluorescence intensity of the different liposome fractions by flow cytometry.

Conjugation of antibodies to liposomes. Anti-digoxigenen F(ab)2-fragments (1 mg/ml in carbonate buffer, pH 8.5) were modified with 2-iminothiolane by incubation for 1 h at room temperature. The product was isolated by gel chromatography on a Sephadex G20 column (PD10, Pharmacia), equilibrated with PBS. The modified protein was stored at -20°C until used. For conjugation, the liposomes were incubated with the modified protein at a final concentration of 0.5 mg/ml and gently stirred for I h. Unconjugated protein was

then removed from liposomes by gel chromatography on Sepharose CL-4B (Pharmacia).

Cells. Peripheral blood mononuclear cells from normal donors were isolated from buffy coats by density centrifugation on a Ficoll Paque (Pharmacia) gradient.

Staining of cells. Staining with antibodies was performed in PBS/0.5% BSA/0.02% NaN3 at 4°C for 10 min followed by two washing steps. The final concentration of CD19 and CD25 antibodies and isotopic control antibody was 1-2 μg ml. Anti-Dig fluorescein-conjugates were used at a concentration of 5 μg/ml. Liposome staining was performed under similar conditions, but for 30-60 min, with gentle agitation and followed by three washing steps. The optimal concentration of each liposome preparation was determined by titration.

Magnetic cell separation. Magnetic cell sorting was performed with the MiniMACS System (Miltenyi Biotec GmbH). The MACS technology has been described in detail elsewhere (Miltenyi et al. (1990) Cytometry 11:231). Briefly, cells were applied to the column in a volume of 0.5 ml of PBS/BSA/NaN3, followed by washing with 0.5 ml of buffer. The effluent was collected as a negative fraction. The column was washed with 0.5 ml of buffer. The positive cells were then collected in 1 ml PBS/BSNNaN_3, after the column had been removed from the magnet. The cells were counted and the recovery was determined from the number of positive cells in the original and the positive fraction.

Flow cytometry. For flow cytometry a FACScan and FACScan research software were used (Becton Dickinson & Co). Propidium iodide (1 μg/ml) was used for exclusion of dead cells. A lymphocyte gate was set by forward and sideward light scattering parameters. For evaluation of data CELLQuest software (Becton Dickinson & Co.) was used.

Results

Liposome preparation and analysis of the liposome size distribution. The percentage of liposomes containing at least one magnetic particle was analyzed by measuring the amount of fluorescein recovered in the various fractions after

extrusion and magnetic filtration. Filtering through membranes with pore sizes ranging from 0.8-0.2 μm decreased the amount of magnetic liposomes from 80-90 down to 50% of total fluorescein encapsulated in liposomes. With a pore size of 0.4 pm, around 70% of fluorescein was recovered in the magnetic fraction.

The size distribution of the liposomes was estimated by measuring the fluorescence intensity of single liposomes by flow cytometry before and after magnetic filtration. The small size of the liposomes did not allow their separation from background signal according to light scattering parameters. Thus, the measurement was triggered on green fluorescence (530 $\pm$ 15 nm). Before separation, a large number of vesicles apparently do not reach the trigger threshold level, which was set to eliminate electronic background, i.e. the sensitivity of the instrument is too low to resolve the whole liposome population from background. In contrast, the fraction of magnetic liposomes eluted from MACS column forms a distinct population, with a peak well above the trigger threshold. The coefficient of variation (c.v.) is markedly reduced and the mean florescence (m.f.) intensity is at least doubled. Thus, small liposomes have been excluded efficiently from the LUV preparation by magnetic filtration. We termed the resulting fraction magnetofluorescent liposomes (MF-LUVs).

Evaluation of MF-LUVs. The MF-LUVs were conjugated to anti-digoxigenin antibodies and tested for their ability to detect rare antigens and allow sorting of cells accordingly. To this end rare antigenic epitopes for anti-digoxigenin antibodies were created on the surface of human B cells by mixing a CD19-digoxigenin-conjugate (CD19Dig) with CD19 phycoerythrin (CD19PE) at various molar ratios and staining of human peripheral blood leukocytes (PBLs) with these mixtures.

Table 1
Mean Fluorescence Values of Sub-populations

	CD19PE/Dig- 10:0	CD19PE/Dig+ 10:1	CD19PE/Dig- 100:1	CD19PE/Dig+ 100:1
Anti-Dig FITC	2.7	5.3	n.d.	n.d.
Anti-dig liposomes	2.6	5660.7	3.4	1461.1

The CD19PE served as an internal control for specificity and sensitivity, while the CD19Dig allowed comparison of an anti-Dig fluorescein-conjugate with anti-Dig MF-LUVs. The staining of anti-Dig liposomes and anti-Dig fluorescein was compared for a molar CD19PE to Dig ratio of 10:1. Mean fluorescence intensities are summarized in Table 1. Staining with fluorosceinated anti-Dig gave a 2-fold increase in mean green fluorescence of CD19PE positive cells, compared to unstained control cells. Due to the coefficient of variation (c.v.) of the stainings, this increase in m.f. intensity was not sufficient to separate populations of positive and negative cells optically according to green fluorescence. In contrast, staining with MV-LUVs could completely separate the two populations. Mean fluorescence of liposome-stained CD19PE+ cells was increased more than 1000-fold compared to anti-Dig fluorescein-stained cells, while the background staining of both anti-Dig reagents was about the same. In another experiment, even at a 100:1 ratio of CD19PE to CD19Dig, positive and negative cells were clearly separated by liposome staining, with an m.f. intensity of about 400-times above background. About 1% of the CD19PE-negative cells are stained with anti-Dig liposomes. This is the same background as that of control cells not stained with CD19Dig but with anti-Dig liposomes. As a high control for the CD19Dig conjugate, cells were stained with pure CD19Dig (2 μg/ml) and anti-Dig fluorescein. Both populations are then very well separated from each other.

Limit of sensitivity. The maximum sensitivity of the MF-LUVs was estimated by increasing the molar ratio of CD19PE over CD19Dig on the PBLs and staining with anti-Dig liposomes. Staining with MF-LUVs is extremely sensitive. Even very few liposomes bound to a single cell (10^3, as determined by fluorescence microscopy and calculated from m.f. intensity of the magnetic liposome fraction) provide sufficient signal intensity to separate the cell from background in flow cytometry. However, at titers higher than 100:1 the staining was no longer quantitative, i.e. not all CD19+ B cells were stained with liposomes. This reflects the kinetics of staining with particles. Compared to antibody molecules the binding of antibodies conjugated to particles is rather slow. Even under optimized staining conditions, i.e. gentle agitation and prolonged incubation times of several hours, the staining is not completely saturating. This

may lead to an all or nothing situation in the case of extremely few antigenic determinants, where some cells are clearly labeled above background, with only or two liposomes bound, whereas other positive cells have not bound any liposome for statistical reasons.

MF-liposomes for magnetic cell separation. It is obvious that the enormous fluorescence intensity provided by MF-LUVs can be used for efficient fluorescence-activated cell sorting. However, magnetofluorescent liposomes can also be used for efficient separation of labeled from unlabeled cells by high gradient magnetic cell sorting with MACS. CD19+ cells were isolated quantitatively by MACS, such that the negative fraction contained only 0.2% CD19+ cells and the purity of positive cells is greater than 96%. From the absolute numbers of cells in the various fractions a recovery of 70-80% of CD19+ cells in the positive fraction was estimated.

Expression of IL2-receptor a-chain (CD25) on a human peripheral blood lymphocytes. CD25 is a marker which has been reported to be expressed at very low level on resting lymphocytes. The expression is strongly upregulated upon activation of the cells. Staining of PBMCs with CD25Dig and anti-Dig fluorescein results in dim staining of a small subpopulation of cells of the CD3+ or CD19+ compartment (Table 2). The exact size of this subpopulation cannot be determined due to the lack of optical separation. For statistical evaluation, markers can be set with the isotype control defining the boundary between positive and negative cells, an approach which will give grossly false values for overlapping populations. About 12% of T cells and 23% of B cells appear to be positive according to this statistical threshold, with an approximately 4-fold increase in m.f. Staining with anti-Dig liposomes strongly increases the signal to noise ratio. The m.f. intensity of positive cells is increased at least 20-30 -fold compared to anti-Dig fluorescein staining. Due to the improved staining, the population of positive cells is clearly separated from negative cells. Now clear-cut statistical markers can be defined and it becomes obvious, that the actual percentage of positive cells is 17% and 40%, respectively. The isotype controls show only 1% or less of unspecifically stained cells. Compared to the staining of CD19 shown before, the m.f. of the positive cells is within the range of

quantitative staining. The overall increase in mean fluorescence intensity compared to conventional staining is less than in the model system shown before. One explanation would be that the m.f. of the anti-Dig fluorescein staining is overestimated due to the overlap of the populations. The c.v. of the liposome-labeled population is relatively high. This might be due to the heterogeneous expression of CD25 on the target cells. It could also result from the low numbers of liposomes bound to a single cell and the intense signal per liposome. Nevertheless the strong signal intensity allows clear discrimination of positive and negative cells.

Table 2

Mean fluorescence intensity values (m.f.) of indicated subpopulations

	CD3+CD25-	CD3+CD25+	CD19+CD25-	CD19+CD25+
Anti-Dig FITC	2.3	31.3	2.6	21.2
Anti-dig liposomes	2.3	962.6	2.8	947.4

To overcome the problem of detecting cellular markers present in low copy numbers, large particles like florescent latex beads or fluorescein-filled liposomes conjugated to antibodies have been used for specific labelling of cells and signal enhancement in flow cytometry. Liposomes can be loaded easily in parallel with various reagents like fluorescent dyes and magnetic beads and, in addition, the flexible membrane provides higher mobility of bound antibodies, compared to latex particles. However, the enhancing effect obtained with conventional liposomes for detection of rare antigens is rather low. The true potential of liposomes for quantitative detection of rare antigens and increasing the signal to noise ratio has not previously been demonstrated.

The analysis of the fluorescence intensity of various liposome preparations in the present experiment revealed that LUVs produced by standard methods of membrane extrusion and reverse phase evaporation contain a dominant fraction of small vesicles. Since small particles have higher diffusion rates and thus faster binding kinetics, those particles can block binding of the larger vesicles and thus reduce the potential enhancing effect of staining. The fast and efficient procedure

to remove small vesicles from the preparation of LUVs described herein yields liposomes with a substantially homogeneous size distribution. Small vesicles are depleted due to their inability to include superparamagnetic particles by high-gradient magnetic sorting. The resulting LUVs provide a dramatic increase in signal-to-noise ration of immunofluorescence. With regard to their handling, magnetic liposomes show additional advantages. Their size distribution can easily be measured by flow cytometry. Within minutes this gives a direct estimate of the real parameter of interest, namely the fluorescence intensity per vesicle. In the same way, long-term stability of the liposomes can be analyzed. Due to their magnetic label it is easy to separate them from unconjugated protein or unentrapped dye, or to concentrate them from dilute suspensions.

The above results demonstrate that mean fluorescence intensity can be enhanced up to 1000-fold with anti-Dig liposomes, compared to conventional staining with anti-Dig FITC. Furthermore, quantitative separation of positive from negative cells is obtained for at least 10-20 times lower concentration of Dig per cell. At even lower concentrations a strong positive signal is still obtained but the staining is no longer quantitative and not all positive cells are labeled. In an application of biological significance, it was shown that a distinct subpopulation of human B and T lymphocytes express CD25, the high affinity receptor for IL2.

Due to the enhanced fluorescence intensity provided by liposomes, populations overlapping in traditional immunofluorescence can be resolved into distinct populations, which makes a correct statistical evaluation possible. The results clearly demonstrate that the magnetofluorescent liposomes are not only useful for signal enhancement, but also allow the detection of subpopulations expressing low numbers of a given antigen. Although the c.v. of staining is increased compared to conventional immunofluorescence, resolution of staining is high especially for homogeneously expressed antigens as is the case for CD19. But even CD25, which is expressed very heterogeneously on resting lymphocytes, can be stained by MF-LUVs with sufficient optical separation for clear discrimination of positive and negative populations. In all situations where the detection of at least some positive cells is sufficient, e.g. expression cloning, separation and analysis of cells positive for a single antigen such as Thy-1 or

surrogate light chain, the detection of tens of hundreds of molecules per cell is possible, making the specificity of the antibody the limiting factor.

In addition to high specificity and sensitivity, magnetofluorescent liposomes also offer the option to isolate the labeled cells by magnetic cell sorting. The simultaneous labelling is especially important in case of low numbers of antigenic epitomes on a single cell to avoid mutual inhibition of magnetic and fluorescent label.

Example 2.

Detection of IL-10 and IFNγ, on the Surface of T cells

Materials and methods

Mice and cells. BALB/c J mice (8-12 weeks) were obtained from Bomholtgard Breeding and Research Centre LTD (Ry, Denmark). Mice transgenic for the DO11.10 ab-TCR (OVAtg) were a gift of Dennis Y. Loh. Murine spleen cells (sc) were isolated and in some experiments depleted of CD5+ T cells with the high-gradient magnetic cell separation system MiniMACS (Miltenyi Biotech GmbH, Bergisch Gladbach, German). The purity of CD8-SC was higher than 99.9%. Human PBMC were prepared from blood samples of healthy donors by FicoII-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

In vitro stimulation of human and murine T cells. Cells were cultured in complete RPM1 1640 (Gibco BRL, Grand Island, NY) containing 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.3 mg/ml glutamine, 10 mM 2-ME and 5% FCS (PAA, Linz, Austria) at 1-2 x 10^6 cells/mi. Staphylococcus aureus enterotoxin B (SEB; Sigma, St. Louis, Mo) was used at 2 μg/ml. In the ab TCR transgenic DO11.10 mice the antigenic peptide of ovalbumin OVA 323-339 (Neosystem S.A., Strasbourg, France) was used at 0.3 μM. Supernatant of the murine fibroblast cell line NIH 3T3, transfected with murine IL-4 cDNA (3T31L4) was added at 1-20% (vol/vol) and recombinant murine IL-12 was added at 100 U/ml, as indicated. After 6-7 days of SEB or OVA culture, cells were harvested and restimulated with PMA (Sigma) at 5 ng/ml and ionomycin (Sigma) at 1 μg/ml for 4-48 hours. In some experiments, brefeldin A (Sigma) was added at 5 μg/ml 2 hours before fixation of the cells.

Antibodies and flow-cytometry. The following rat anti-mouse cytokine mAB were used: anti-IL-2 S4B6, anti-IL-4 11B11 and 24G2, anti-IL-5 TRFK4 and TRFK5, anti-IL-10 JES5-2AT, SXC1 and SXC2, anti-IFN-γ R4642 and AN18.17.24. The following mouse anti-human cytokine mAb were used: anti-IFN-γ 45-15 (provided by Sefik Alkan, Ciba-Geigy, Basel) and GZ04 (Boehringer Mannheim, Mannheim, Germany). Antibodies against surface markers: murine CD4 GK1.5/4 and CD8 53-6.7; PE conjugated anti-human CD4 and CD8 were purchased from Beckton Dickinson (San Jose, CA).

For flow cytometry, a FACScan and CELLQuest research software were used (Becton Dickinson). For fluorescence activated cell sorting a FAC5Star+ and LYSIS II software (Beckton Dickinson) were used. Propidium iodide (PI; 1 mg/ml) was used for exclusion of dead cells.

Magnetofluorescent liposomes. Sheep anti-digoxigenin (DIG) Fab fragments (Boehringer Mannheim) conjugated to magnetofluorescent liposomes were prepared as described previously. Briefly, a mixture of lipids (Lipold KG, Mannheim; cholesterol purchased from Sigma) molar ratio DPPC:Chol:DPPG:PDP-DPPE=45:40:10:5) dried in a glass vessel was hydrated at 65°C with PBS containing 10 mM CF and super-paramagnetic microparticles (about 50 nm in diameter; Miltenyi Biotec). The suspension was submitted to several freeze-thaw cycles and extruded five to ten times through filters of 800nm and then 400 nm pore size (Millipore, Eschborn, Germany). Free magnetic particles were removed from liposomes by centrifugation over a Ficoll (Pharmacia) density gradient. Magnetic liposomes were then isolated by high gradient magnetic field filtration with MACS (Milteny Biotec).

Sulfhydryl groups were introduced in anti-digoxigenin $F(ab)_2$-fragments by incubation with 2-iminothiolane. The modified protein was purified by gel chromatography on Sephadex G20 columns (PD10, Pharmacia). For conjugation, the liposomes were incubated with the modified protein at a final concentration of 0.5 mg/ml. Unconjugated protein was the removed from liposomes by gel chromatography on Sepharose (CL-4B (Pharmacia).

Surface staining of Cytokines. Surface staining with antibodies was done in PBS/0.5% BSA/0.02% NaN3 at 4°C for 10 minutes followed by two washing

steps. Unspecific binding sites were blocked by preincubation of cells with anti Fcγ,-receptor antibody 2.4 G2 75 at 20 μg/ml or purified Ig (Nordic, Tiburg, Netherlands) at 0.5-3 mg/ml. The final antibody concentration of anti-cytokine antibodies and isotype control antibody was 1-5 μg/ml. Liposome staining was performed for 30 minutes with gentle agitation, followed by two washing steps at 300g for 10 min.

Intracellular staining of cytokines. Cells were fixed in 2% formaldehyde (Merck, Darmstadt, German) as described previously. For staining, 10^5-106 fixed cells were incubated with DIG- or NP-conjugated rat anti-mouse or mouse anti-human cytokine mAb (51 μg/ml) in PBS/BSA/NaN3, containing 0.5% saponin (saponin buffer, Sigma) for 15 min at RT. Cells were washed once or twice with saponin buffer and stained with PE-conjugated sheep anti-DIG Fab fragments (Boehringer Mannheim) or PE-conjugated mouse anti-NP S43-10 in saponin buffer for 20 min at RT. Cells were washed once or twice with saponin buffer. Finally, cells were resuspended in PBS/BSA/NaN3 for flow cytometric and microscopic analysis.

IL-4, IL-5, IL-10 and IFN-7 ELISA. Concentrations of cytokines in the culture supernatants were measured by sandwich ELISA as described before using mAB 11B11, TRFK5, SXC2 and R46A2 as coat, and biotinylated 24G2, TRFK4, SXC1 and AN18.17.24 for detection of IL-4, IL-5, IL-10 and IFN-7, respectively.

Results

Detection of IFN-γ, on the surface of activated T cells in mouse and man. Murine spleen cells were activated in vitro either with the bacterial superantigen SEB or, in case of cells from αβ T cells receptor transgenic DO11.10 mice (Murphy et al. (1990) Science 250:1729), with the specific ovalbumin (ovA) peptide. Human PBMCs were stimulated in vitro with SEB. In both systems, cells were stimulated for up to 7 days with or without addition of exogenous IL-4 and then restimulated with PMA and ionomycin for 4-48 hours, in some experiments in the presence of brefeldin A. At different time points of primary or secondary stimulation, live cells were stained by immunofluorescence

for cytokines on the cells surface, and fixed and permeabilized cells were stained for intracellular cytokines.

Surface-associated IFN-γ was found on cells of cultures of primary, as well as secondary stimulation. Data is shown in Figure 2 and Figure 3. Figures 2A to 2E are plots showing the staining of IFN-γ on the surface of live murine CD4 (Figure 2c) and CD8 (Figure 2E) T cells after 3 days activation with SEB and gated on activated blast cells (Figure 2A). Specificity of staining was confirmed by negative control staining with an.isotypic antibody (Figure 2B) and specific inhibition of the staining with an excess of soluble IFNγ (Figure 2D). Expression of surface IFN-γ was restricted to large, activated blastoid cells, as had been shown before for intracellular IFN-γ. Surface associated IFN-γ was found on activated murine as well as on activated human T cells and it was detectable on both CD4+ and CD8+ T cells. Figures 3A and 3B show surface IFN-γ, staining on human cells after 6 days SEB culture and 5 hours restimulation with PMA/ionomycin (Figure 3B). Specificity of staining is confirmed by isotypic control antibody (Figure 3A).

Only some of the activated T cells stained for surface IFN-γ, as previously shown for intracellular IFN-γ, (Assenmacher et a/. (1994) Eur. J. Immunol. 2412:1097). Surface associated IFN-γ, was found on superantigen as well as on antigen activated T cells. While surface bound IFN-γ was difficult to detect by conventional immunofluorescence, it was clearly detectable by "enhanced" immunofluorescence using indirect staining with magnetofluorescent liposomes. Specificity of staining for surface IFN-γ was controlled by isotope matched control antibodies, by combined staining with different antibodies against IFN-γ, like rat anti-mouse IFN-γ, mAb AN18.17.24 and rat anti-mouse IFN.-γ, mab R46A2, and by inhibition of surface IFN-γ staining with added IFN-γ.

To analyze whether surface IFN-γ, is a distinct feature of IFN-γ producing cells, activated T cells, stained for surface IFN-γ, were isolated by MACS and FACS and then either fixed, permeabilized and stained for intracellular IFN-γ or taken into culture and analyzed for secreted IFN-γ, by ELISA. Due to saponin-permeabilisation, surface staining of IFN-γ, with liposomes is almost completely lost, making it impossible to directly correlate surface vs. intracellular IFN-γ.

Staining of surface vs. intracellular IFN-γ was controlled by fluorescence microscopy.

Figures 4A to 4D show the correlation of surface and intracellular IFN-γ expression by the same cells. Naive OVA-TCRtg/tg T cells were activated by the specific OVA peptide + IL-12 for 6 days and restimulated with PMA/ionomycin for 5 hours. The cells were then stained for surface IFN-γ expression (Figure 4A) and sorted by MACS or FACS. The purified populations (purity 97-99%) were then fixed and analyzed for intracellular IFN-γ expression (Figure 4B; Figure 4C; Figure 4D). The data show close correlation of surface and intracellular IFN-γ expression at the single cell level. Among human CD8-PBMC, activated for 6 days with SEB, restimulated with PMA and ionomycin for 5 hours, stained for surface IFN-γ and sorted by FACS, most surface IFN-γ positive cells also stained for intracellular IFN-γ (86% compared to 19% in unseparated cells), while only few (7%) of the surface IFN-γ negative cells stained for intracellular IFN-γ. Among murine CD4+ T cells, activated for 6 days with SEB + IL-4, restimulated with PMA and ionomycin for 4 hours and then sorted for surface IFN-γ, the surface IFN-γ positive cells secreted 23 times more than the IFN-γ negative cells, when cultured further for 1 day after sorting.

In primary SEB culture, expression of surface and intracellular IFN-γ was detectable with nearly identical kinetics on and in activated CD4+ murine T cells, respectively, with a peak of expression around day 2 to 3. The transient kinetics of expression of surface and intracellular IFN-γ might also explain why 86% but not all of FACS sorted surface IFN-γ+ cells and 7% but not none of FACS sorted surface IFN-γ cells stained for intracellular IFN-γ. While some surface IFN-γ+ cells might have finished IFN-γ production during sorting before fixation, some surface IFN-γ cells might have started to produce IFN-γ short before fixation.

Surface IFN-γ is expressed on IFN-γ, but not IL-4 or IL-5 producing cells. Stimulation of murine and human T cells with SEB induces high number of IFN-γ producing T cells, but almost no IL-4 producing cells. By addition of exogenous IL-4 to primary SEB cultures of murine spleen cells (SC), the number of IFN-γ producing cells is reduced and IL-4 expressing cells are induced. Simultaneous

intracellular staining for the two cytokines showed no detectable cells co-producing IFN-γ and IL-4 above the limit of detection (0.5%).

Figure 5 is a bar graph showing cytokine secretion by murine T cells sorted for surface IFN-γ expression. Murine spleen cells were activated with SEB + IL-4 (to reduce the number of IFN-γ producing cells and increase the number of IL-4 producers) for 6 days, and restimulated for 4 hours with PMA/ionomycin. After staining and sorting for surface IFN-γ, the cells were cultured for a further 24 hours. After that time, supernatants were analyzed by ELISA for cytokine contents. IFN-γ is almost completely produced by the surface IFN-γ+ population whereas the amount of IL-4 and IL-5 is reduced in this fraction. Relative detection limits were 0.01, 0.04, 0.04 and 0.001 for IL-4, IL-5, IL-10 and IFN-γ ELISAs, respectively. The surface IFN-γ+ fraction secreted 3-4 times less IL-4 and 3-4 times less IL-5 than the negative fraction. IL-10 was secreted to a similar amount by both fractions. This is in accordance with previously described results (Assenmacher, supra.), where cells co-expressing IFN-γ and IL-4 were not detectable, but about 50% of the IFN-γ producing cells co-expressed IL-10. The data show that surface IFN-γ is independent from the status of activation or cytokine secretion in general of the cells but only depends on IFN-γ production. Thus, it is possible to specifically sort IFN-γ secreting cells out of a mixed population of cytokine producing cells.

Surface IFN-γ is also detectable on T cells which lack the natural IFN-γ receptor. The percentage of intracellularly positive cells correlates with the number of cells positive on the surface. Figures 6A to 6D show the expression of surface and intracellular IFN-γ by murine IFN-γ receptor negative T cells. IFN-γ receptor -/- spleen cells were activated for 6 days with SEB and restimulated with PMA/ionomycin. Subsequently, the cells are stained for intracellular (Figure 6B) or surface (Figure 6D) IFN-γ. Negative controls in Figure 6A and Figure 6C were staining without anti-IFNγ antibody.surface IFN-γ, is also detectable on T cells which lack the natural IFN-γ receptor and that the percentage of intracellularly positive cells correlates with the number of cells positive on the surface. Sorted cells were cultivated for 24h and supernatants analyzed for IFN-γ

content by ELISA. The surface IFN-γ+ cells secrete IFN-γ, whereas the surface IFN-γ, cells produce very little.

Previously, IFN-γ, like most other cytokines, was known to occur only as a secreted protein. Secreted IFN-γ is a homodimer without a trans-membrane region, and so it is unclear how it is attached to the cell surface. Immunofluorescence staining of cytokines bound to their receptor has been demonstrated for IL-2 on cell lines, however this explanation is very unlikely, since IFN-γ receptor negative T cells have detectable surface IFN-γ.

IL-10 can also be detected on the surface of activated T cells. Figures 7A to 7C show the expression of surface IL-10 on the surface of activated murine T cells. Murine spleen cells were activated for 6 days with SEB + IL-4 (to induce high amounts of IL-4) and stained for surface IL-10 (Figure 7A). Specificity of staining was confirmed by negative control staining without anti-IL-10 antibody (Figure 7B) and specific inhibition of staining by an excess of soluble IL-10 (Figure 7c).

Murine OVA-TCRtg/tg T cells were activated by the specific OVA peptide + IL-4 and anti-IL-12 for 6 days and restimulated with PMA/ionomycin for 4 hours. The cells were then stained for surface IL-10 expression (Figure 8A) and sorted by MACS or FACS. The purified populations (purity 95-99%) were then fixed and analyzed for intracellular IL-10 expression (Figure 8B; Figure 8C; Figure 8D). (Remaining liposomes on the x-axis of the dot-plot are surface bound liposomes which are not completely destroyed by the intracellular staining procedure). The data show close correlation of surface and intracellular IL-10 expression on the single cell level.

Murine spleen cells were activated with SEB + IL-4 for 6 days, and restimulated for 4 hours with PMA/ionomycin. After staining and sorting for surface IL-10, the cells were cultured for further 24 hours. After that time, supernatants were analyzed by ELISA for cytokine contents. As expected, IL-10 is strongly enriched in the surface IL-10+ population as well as IL-4 (both cytokines are known to be coexpressed by the same cells), whereas the amounts of IL-2 and IFN-γ are unchanged in this fraction. Figure 9 is a bar graph showing cytokine secretion by murine T cells sorted for surface IL-10 expression.

It is clear from the above results that the subject invention provides for highly sensitive, specific, fluorescent labeling. The ability to select for large magnetofluorescent liposomes provides a significant improvement over heterogeneous liposome staining reagents. The use of a sensitive staining method permits the detection of certain cytokines on the surface of lymphocytes. The staining methods provide sufficient resolution to allow viable and functional intact isolation by cell sorting. This opens new possibilities for the analysis, diagnosis and prospective cellular therapy of immune reactions with regard to the functional diversity of lymphocytes.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A composition of magnetofluorescent liposomes, having a diameter of from about 100 to 500 nm, and conjugated to a specific binding agent.

2. A composition according to Claim 1, wherein said specific binding agent is an antibody.

3. A liposome according to Claim 2, wherein said antibody is specific for a hapten.

4. A liposome according to Claim 2, wherein said antibody is specific for a cytokine.

5. A liposome according to Claim 4, wherein said cytokine is interferon γ.

6. A liposome according to Claim 4, wherein said cytokine is IL-10.

7. A method for producing a magnetofluorescent liposome composition substantially uniform in size, the method comprising:

forming said liposomes in the presence of a fluorescent dye and superparamagnetic microparticles, wherein said microparticles have a diameter of from about 50 to 100 nm in size;

extruding said liposomes through a membrane having a pore size of about 400 nm;

selecting by high gradient magnetic selection for liposomes comprising a magnetic particle;

wherein said selected liposomes have a diameter of from about 100 to 500 nm.

8. A method of detecting cells according to a cell surface marker expressed at low levels, the method comprising:

staining said cells with a composition according to Claim 1, wherein said specific binding agent specifically binds to said cell surface marker.

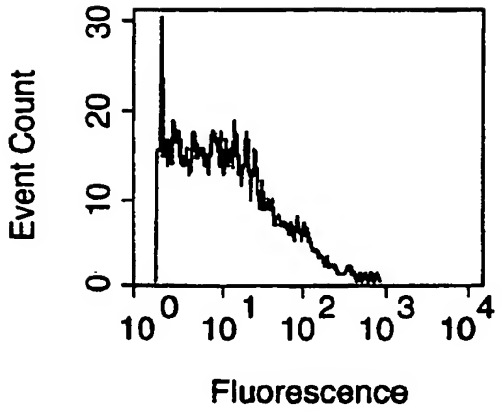


FIGURE 1A

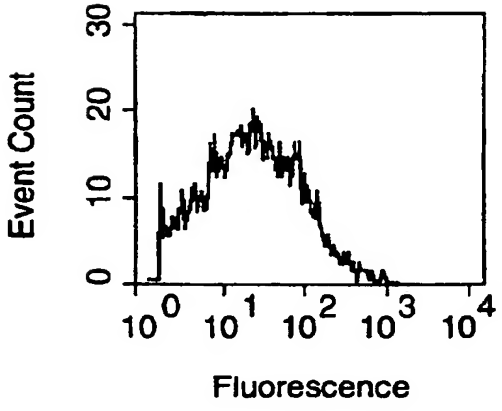


FIGURE 1B

FIGURE 2A

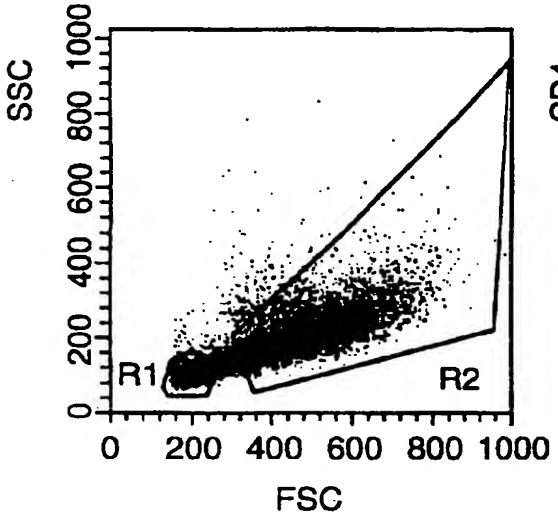
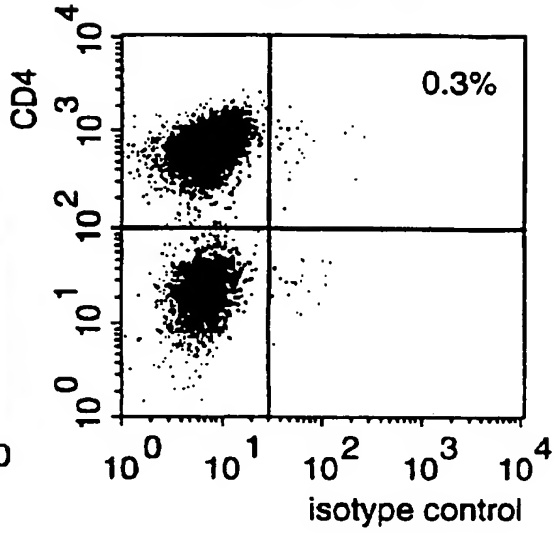


FIGURE 2B



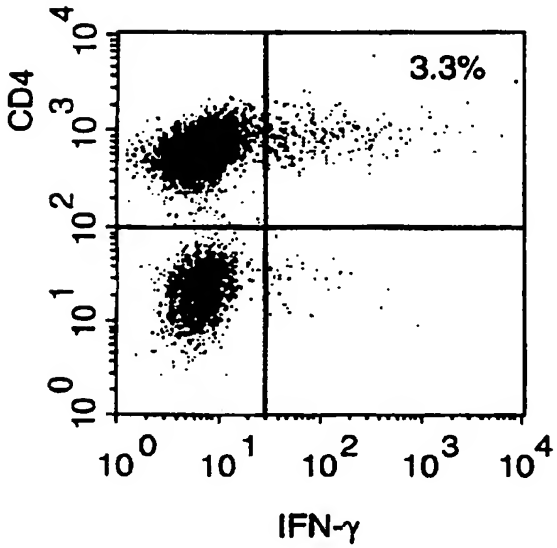


FIGURE 2C

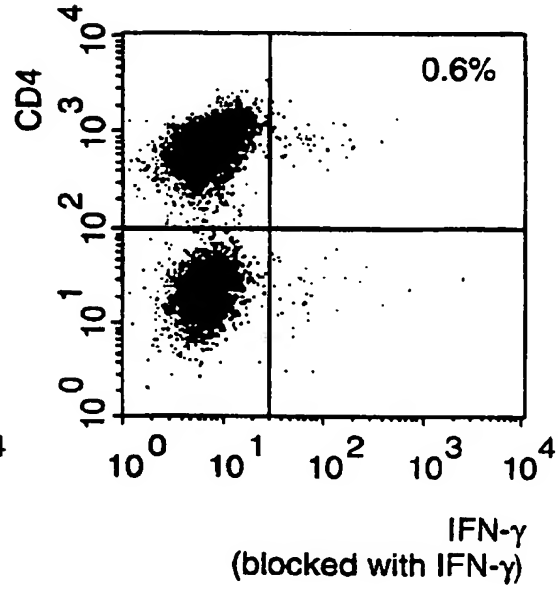


FIGURE 2D

FIGURE 2E

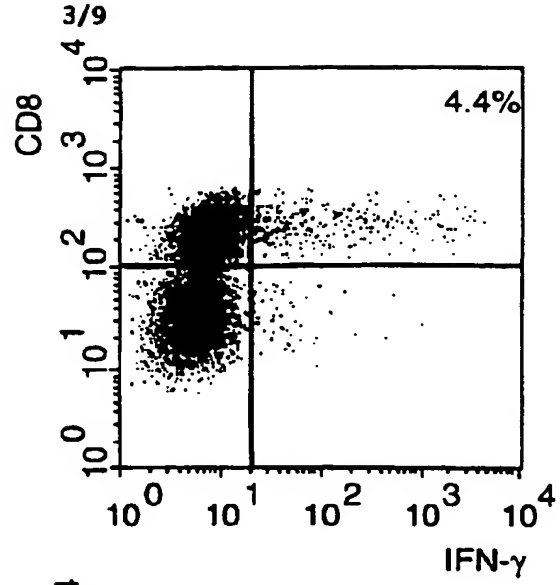


FIGURE 3A

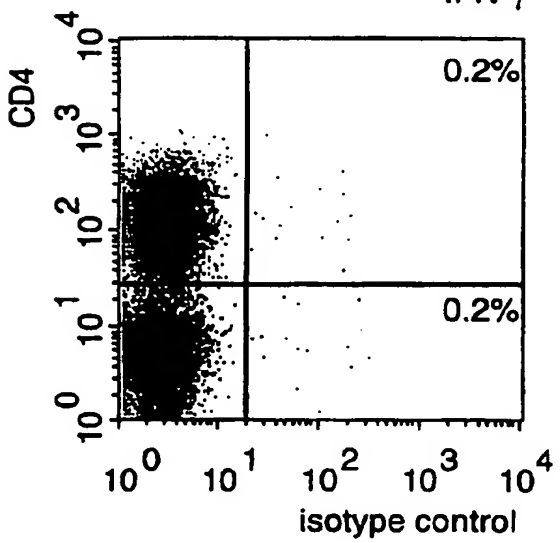


FIGURE 3B

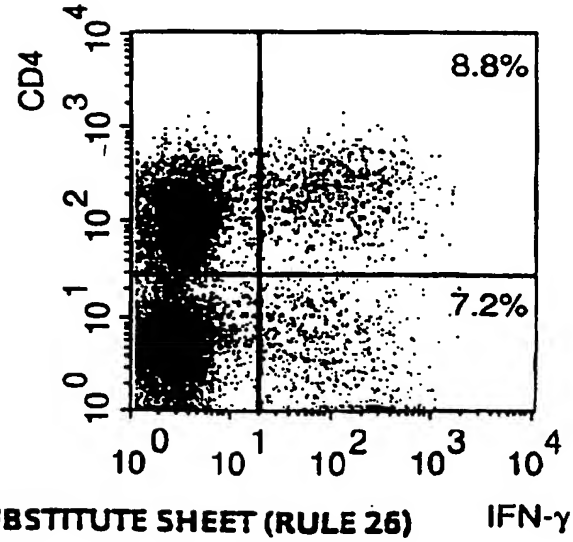


FIGURE 4A

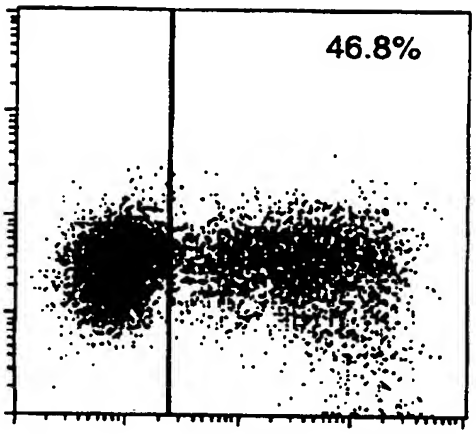
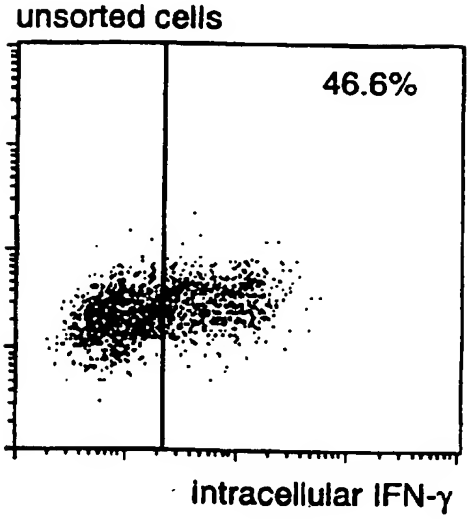
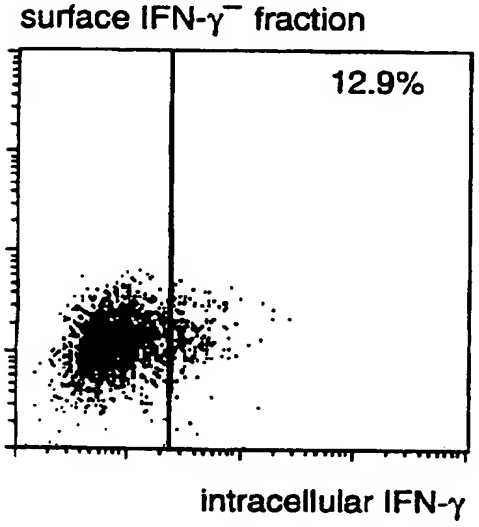


FIGURE 4B





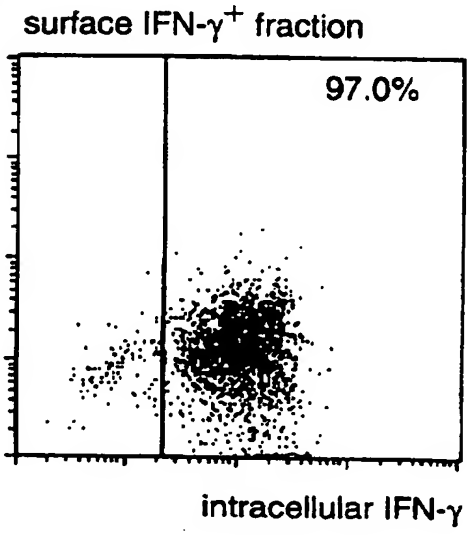
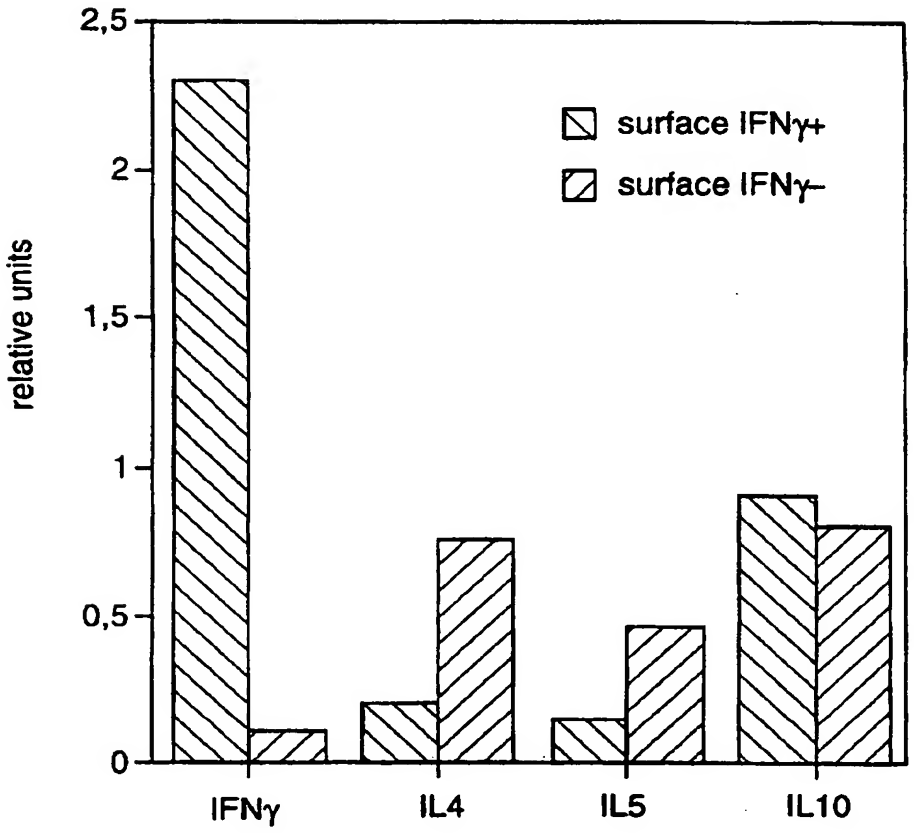


FIGURE 4C

FIGURE 4D

FIGURE 5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1
0,5
0
relative units
surface IFNγ+
surface IFNγ–
IFNγ
IL4
IL5
IL10

FIGURE 6A

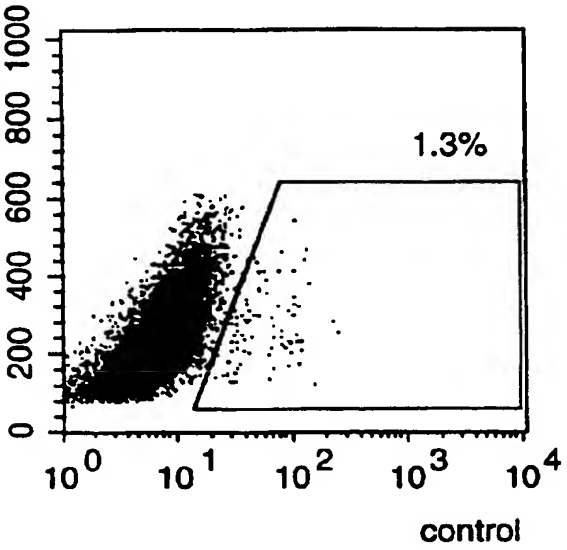


FIGURE 6B

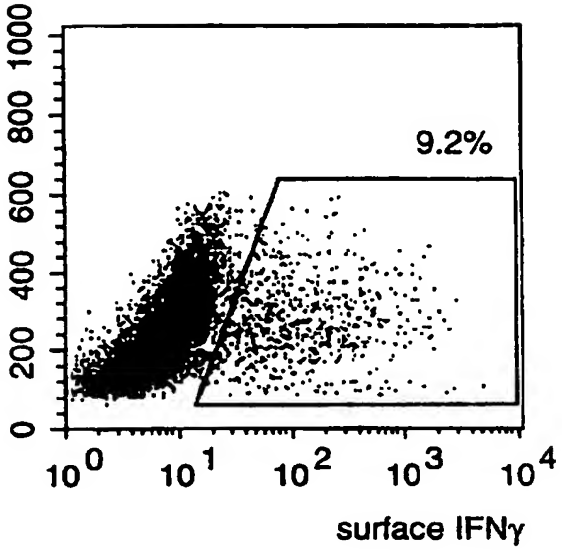


FIGURE 6C

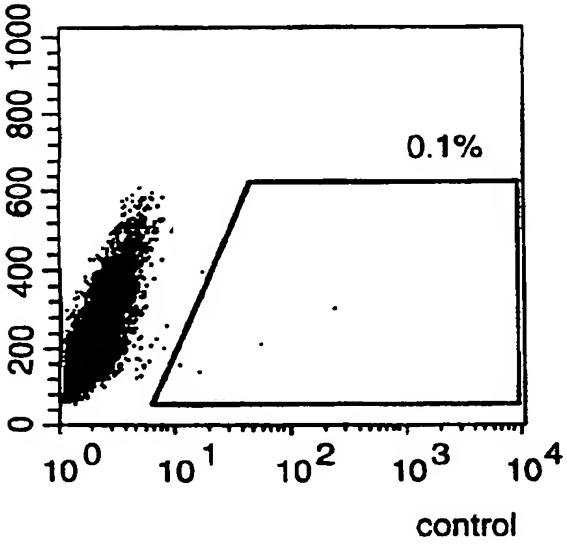


FIGURE 6D

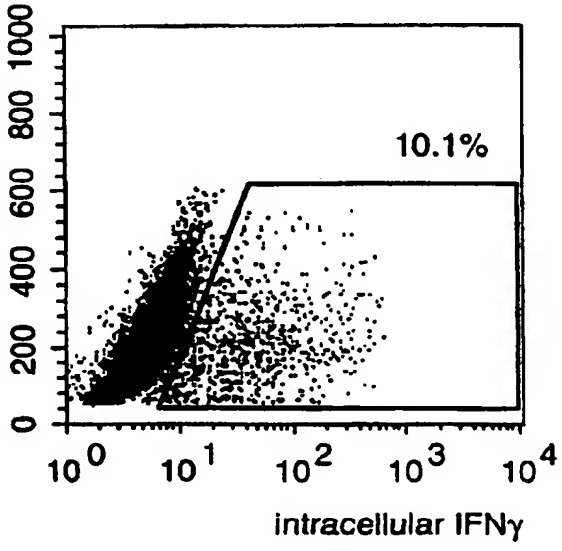


FIGURE 7A

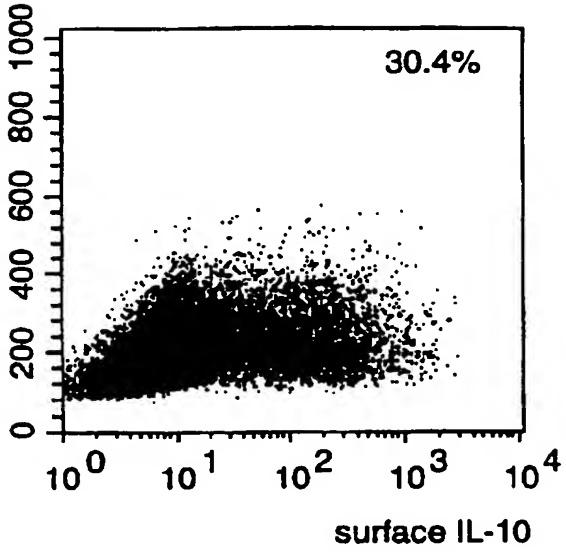


FIGURE 7B

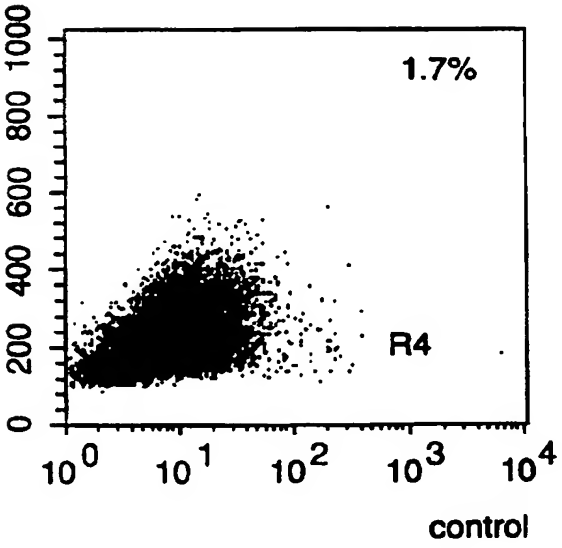


FIGURE 7C

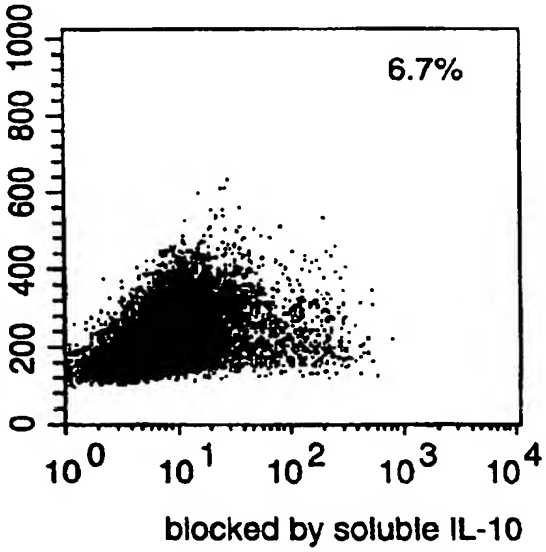


FIGURE 8A

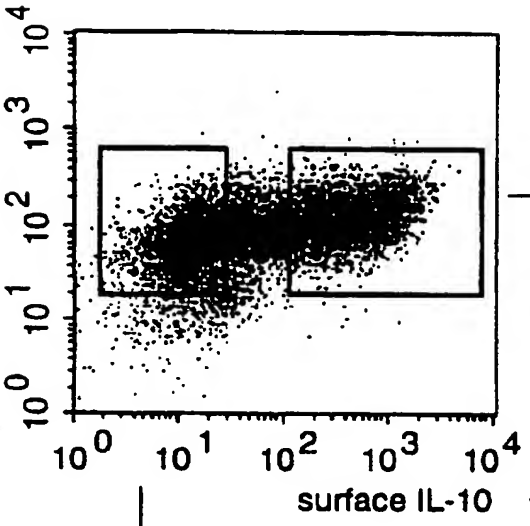
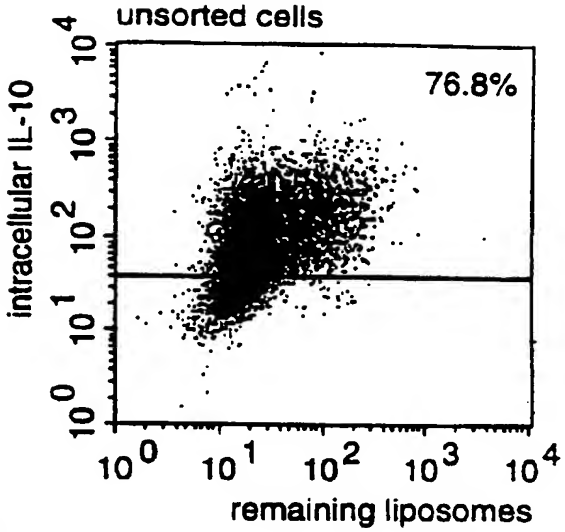


FIGURE 8B



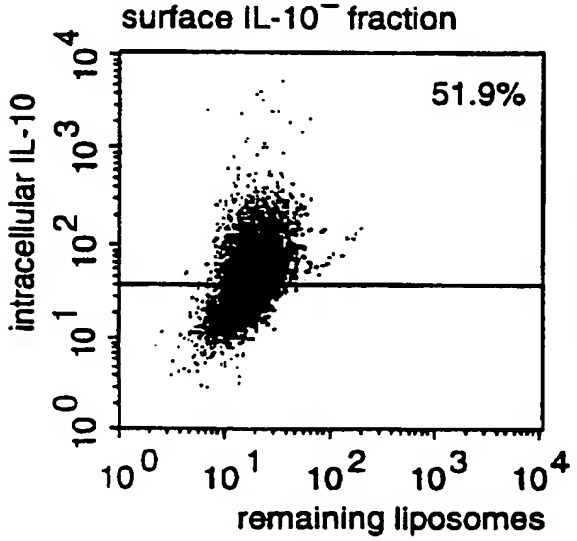


FIGURE 8C

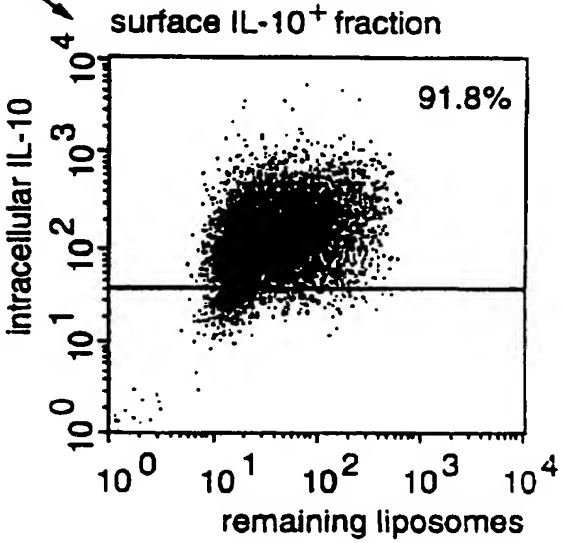
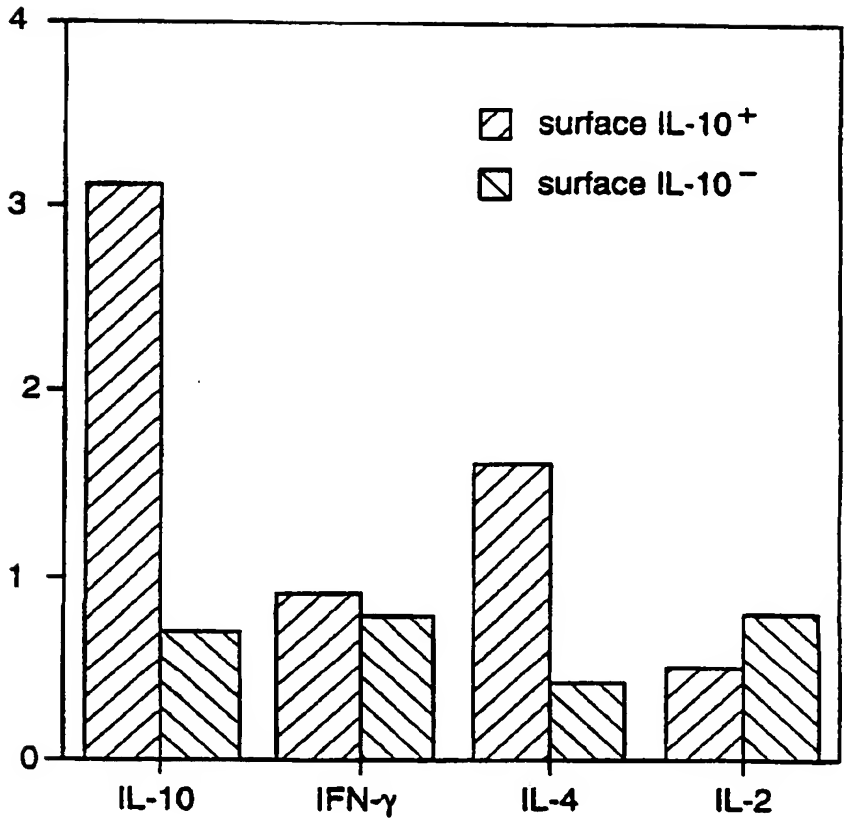


FIGURE 8D

FIGURE 9



INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/12657

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/543 G01N33/569 G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. SCHEFFOLD ET AL.: "Magnetofluorescent liposomes for increased sensitivity of immunofluorescence." IMMUNOTECHNOLOGY, vol. 1, no. 2, 1995, AMSTERDAM NL, pages 127-137, XP004052715 see the whole document ---	1-8
X	US 4 369 226 A (A. REMBAUM) 18 January 1983 see claims 3,9; example 17 --- -/--	1-3

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 November 1997	21. 11. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

Inter[illegible]nal Application No
PCT/US 97/12657

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	M. ASSENMACHER ET AL.: "Fluorescence-activated cytomytry cell sorting based on immunological recognotion" CLINICAL BIOCHEMISTRY, vol. 28, 1995, NEW YORK NY USA, pages 39-40, XP002046269 see page 40, column 2, line 14 - line 37 -----	1-8

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INTERNATIONAL SEARCH REPORT

Information on patent family members

National Application No
PCT/US 97/12657

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 4369226 A	18-01-83	US 4267234 A	12-05-81